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Chemiluminescence Assay for the Detection of Biological Warfare Agents

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DISCLAIMER

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Abstract

A chemiluminescent homogeneous immunoassay and a hand-size multiassay reader are described that could be used for detecting biological materials. The special feature of the assay is that it employs two different antibodies that each bind to a unique epitope on the same antigen. Each group of epitope-specific antibodies has linked to it an enzyme of a proximal-enzyme pair. One enzyme of the pair utilizes a substrate in high concentration to produce a second substrate required by the second enzyme. This new substrate enables the second enzyme to function. The reaction of the second enzyme is configured to produce light. This chemiluminescence is detected with a charge-coupled device (CCD) camera. The proximal pair enzymes must be in close proximity to one another to allow the second enzyme to react with the product of the first enzyme. This only occurs when the enzyme-linked antibodies are attached to the antigen, whether antigen is a single protein with multiple epitopes or the surface of a cell with a variety of different antigens. As a result of their juxtaposition, the enzymes produce light only in the presence of the biological material. A brief description is given as to how this assay could be utilized in a personal bio-agent detector system.

Introduction

Motivation

The fall of the Berlin Wall and the reorganization of eastern European political power has brought a dramatic change in the perception of potential threats to the United States. The fear of nuclear warfare with the Soviet Union has been replaced with the new, and perhaps more probable, threat of biological, chemical, and nuclear assault originating from terrorist groups and hostile rogue governments. This fear has apparently been justified by recent events in the United States (the bombing of the World Trade Center in New York) and around the world (the Persian Gulf War, the bombings of the U.S. embassies in Africa, and the Aum Shinrikyo terrorist activity in Japan). These incidents demonstrate the vulnerability of developed countries to attacks by terrorists and to warfare perpetrated by unethical governments. To respond to this new threat, a national defense strategy is developing in the United States to address issues related to the use of biological, chemical, and nuclear weapons by terrorists against the United States, its citizens and its infrastructure. An important part of this new strategy is development of a variety of new and sensitive BW agent detectors.

Current Status of U.S. Capability

Recognition of the potential threat posed by an attack against U.S. assets either domestically or abroad prompted assessments of the U.S. defensive biowarfare capability. Without question it was found that both military and civilian ability to respond to a biological terrorism event was inadequate and new efforts were necessary to develop policies, procedures, and technology to reduce the potential of an event and to mitigate the enormity of an event should one occur. In this regard, several state-of-the-art technologies have been recognized as indispensable to any

new bioagent detection/identification sensor system. Most prominent among these are polymerase chain reaction (PCR) technology, flow cytometry, mass spectrometry, and immunoassay technology. For rapid and specific detection of biological agents, PCR, flow cytometry, and immunoassay technologies are the most sensitive, rapid, and likely approaches to be implemented as primary detection methodologies. However, while some of these approaches are quite sensitive and specific, they also cannot easily be modified for use outside of the laboratory. The purpose of the technology described in this report is to overcome issues of sensitivity, specificity, and portability that face new detection/identification technology destined for deployment in the field. The assay described in this report is the basis for a robust and compact personal monitor and point-sensor for detecting and identifying biological agents in either indoor or outdoor non-laboratory environments.

Introduction to Immunoassay Technology

The development of immunochemistry in the first part of the 20th century showed early on that antibodies could be used for detecting specific antigens. However, it was from the pioneering efforts of Yalow and Berson and Elkins and their development of radioisotope-labeled antigens that immunoassay technology got its initial boost as a truly analytical method for detecting and quantifying antigens. Since then a broad array of antibody and antigen labels has been developed which has ushered in a complimentary suite of optical methods to measure the antibody-antigen reaction. The most significant new labeling technique, introduced in the 1970's, was the use of enzymes as non-radioactive labels for antibodies [Engvall]. The enzymes, covalently attached to the antibody, were used to catalytically produce easily detectable products. This was the first major advancement in assay technology following the development of radioimmunoassays (RIA) and fostered in a new era of

safe and convenient enzyme immunoassays (EIA) which could be performed by most anyone.

The structure of IgG type antibodies (immunoglobulin type G), the family of antibodies normally used in immunoassays, has the characteristic "Y" or "T" shape (see Figure 1). The arms of the antibodies, the Fab region, are identical having the same amino acid structure and three-dimensional conformation. This identity means that the antigen binding portion at the distal ends of the Fab region binds to the same epitope (antigenic determinant) of an antigen. The two Fab regions are covalently linked to one another at the "hinge" through a disulfide bond, and the pair of Fabs are linked with a second -S-S- bond to the Fc region, the leg of the "Y". Each Fab region is about 45,000 Daltons and the Fc region is nearly 50,000 Daltons. The overall mass of the IgG antibody is roughly 150,000 Daltons with some oligomeric sugars attached to the Fc fragment. The Fab regions are folded sections of protein giving the segment a cylindrical shape with a diameter of 3.5 nm and a length of 6.5 nm. The Fc region has a diameter of 4.0 nm and a length of 5.0 nm.

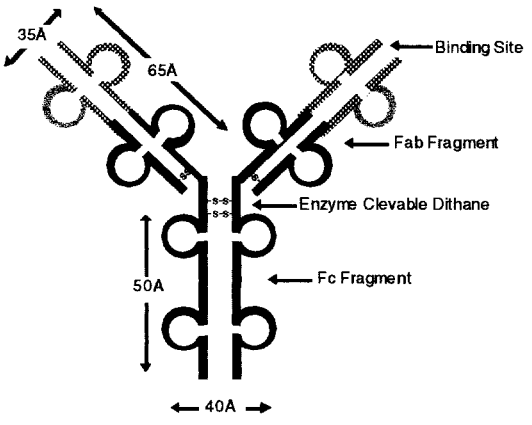


Figure 1. Diagrammatic view of immunoglobulin type G used in most immunoassays. The two Fab fragments are identical and bind to identical epitopes. The Fab fragment is about 45,000 Daltons. The Fc fragment does not bind antigen. The Fc fragment has a mass of 50,000 Daltons. The dithianes can be reduced to separate the individual chains that constitute the Fc and Fab fragments.

The Fc region is not needed for antigen recognition or binding and can be removed with enzymatic cleavage of the disulfide bonds that link it to the Fab chains. However, the location-of-choice for the covalent linkage of enzyme labels to the antibody is often through modification of the sugar groups attached to the Fc region. The chemical reactions required for this linkage are mild and the maximum number of enzymes attached to the antibody in this way is limited to no more than four: there are only two terminal sugar groups available for modification and the reactions that generate the enzyme linkage sites produce two sites from each sugar. This form of selective labeling of the antibody minimizes steric interactions that could occur between the bonded enzyme and the antibody binding site, thereby reducing the potential for affecting the antibody-antigen interaction and altering the antibody affinity and antigen specificity.

The basic reaction of an antibody (Ab) with an antigen (Ag) is shown in Equation 1. At equilibrium the concentration of bound ([Ab-Ag]) and free ([Ag]) antigen is determined and the equilibrium constant K_{eq} is calculated from Equation 2.

$$Ab + Ag \underset{k_r}{\overset{k_f}{\rightleftharpoons}} Ab\text{-}Ag \qquad (1)$$

$$K_{eq} = \frac{[Ab\text{-}Ag]}{[Ab][Ag]} \qquad (2)$$

$$K_{eq} = \frac{k_f}{k_r} \qquad (3)$$

The forward, or binding, rate constant (k_f) and reverse, or dissociation, constant (k_r) are related to the K_{eq} as shown by Equation 3. The forward rate is often a measure of the affinity of the monovalent binding site on the antibody for the antigen, and in the

absence of other factors, K_{eq} is a measure of the intrinsic binding of a site. For bivalent or multivalent antibodies that can attach both binding sites to a single antigen, the total binding is much stronger than for a monovalent site and in this case K_{eq} is a measure of the avidity of the antibody for the antigen.

The initial interaction between the antibody and antigen is diffusion-controlled ($k_{diff} = 10^9$ s^{-1} $mole^{-1}$) but the orders-of-magnitude-lower binding constant (typical range for $k_f = 10^6$ to 10^8 s^{-1} $mole^{-1}$) for the forward reaction in Equation 1 suggests that following the initial collision there is formation of an encounter complex that undergoes rearrangement before establishing the minimum energy antigen-antibody orientation. The specificity of an antibody for a unique antigen is often higher for reactions that exhibit a lower affinity, while antibodies that react with a variety of similar antigens often have much larger values of K_{eq}. This relation, analogous to the issue of kinetic versus thermodynamic control over the course of a reaction, is important to consider when evaluating monoclonal antibodies for use in an assay, as the antibody affinity will influence the ability of the assay to measure a single unique antigen.

Equation 1 shows the overall reaction of the antibody with antigen. In order to make this reaction useful as an analytical or diagnostic tool one must be able to measure the concentrations of the free and bound antigen, or at least determine if antigen is present. This can be done in a number of ways, but there is almost always a separation step required where the bound antibody-antigen complex is separated from the unbound antigen. Typically, radioimmunoassays employ the competitive binding technique which involves developing a standard response curve for a fixed concentration of antibody reacting with a range of known concentrations of a standard antigen solution containing a fixed amount of isotopically labeled antigen. Activity of the bound antigen and free antigen are measured after thoroughly separating the

bound antigen from the unbound. This can be accomplished by selectively precipitating all of the antibody in the solution and carefully washing only the unbound antigen from the medium. The more standard antigen bound by the antibody the less there will be of bound radioactive antigen and more of this labeled antigen will be in solution. From the activity generated by known concentrations of standard antigen, a curve is generated with the radioactivity measured in the separated fractions as a function of standard analyte concentration.

The solution of sample containing the unknown antigen concentration is then divided up and mixed in known proportions with the fixed concentration of labeled antigen. The binding reaction is performed and the unlabeled antigen competes with the chemically equivalent radioactive antigen for the antibody binding sites. An equilibrium is established where the unbound antigen is a mixture of radioactive and unlabeled antigen. Similarly, the bound antigen is a mixture of radioactive and unlabeled antigen. By completely separating the unbound antigen from that of the antibody-complexed antigen, the activity level of the mixed antigen reactions can be compared with the activity of the standard curve and one can determine the concentration of sample antigen in the solution in question.

Assays that use enzymes as labels generate similar standard activity curves, but in this case the activity is a measure of the indicator concentration produced by the enzyme label over a given time. However, instead of separating bound and free antigen, one separates the bound and free enzyme-linked antibody. Generally, an unlabeled antibody that binds the antigen is immobilized to a surface. The immobilized antibody is treated with solutions of known antigen concentration and the binding process is allowed to come to equilibrium. Unbound antigen is completely removed and the antigen bound to the surface is treated with an excess enzyme-

linked antibody specific for the bound antigen. After reaching equilibrium, excess labeled antibody is washed from the surface and the antigen-bound antibody-linked enzyme is treated with substrate to generate an observable signal. The concentration of antigen in the initial standard solutions is a function of the activity of the bound antibody-linked enzyme. The solution of unknown antigen concentration is treated in a similar way and the resulting enzyme activity is compared to the standard curve.

A common transduction scheme employed with EIA is to utilize enzymes that promote changes in the optical properties of the assay media. Generally, these changes are brought about by enzyme-catalyzed reactions that convert transparent substrates into colored, fluorescent, or chemiluminescent molecules. An efficient enzyme used as a label might be capable of generating tens of thousands of optically detectable product molecules each second during the course of the assay. This represents a tremendous enhancement in the observable signal over antibodies labeled directly with absorptive or fluorescent indicator molecules. The directly labeled antibody may have as many as 50 indicator molecules attached to it, whereas an antibody labeled with enzymes could produce indicator molecules on the order of ten thousand times that amount every second over the course of the assay, or generate a similar number of photons from an enzyme-catalyzed chemiluminescent reaction. Each of the three basic transduction methods (absorbance, fluorescence, luminescence) has its particular advantage for a given assay protocol; however, as shown in Table 1, assays based on chemiluminescence generally exhibit higher sensitivity, or lower limits of detection (LOD in moles/L).

The application of immunoassay technology has exploded over the last ten years with development of a wide variety of assay approaches for use in the laboratory, in medical offices, and even for home use. However, even these new

Table 1. Comparison of the Relative Sensitivity of Different Immunoassays.

Transduction Method	Analyte	LOD	Molecules/100μL
fluorescein labeled antibody (fluorescence)	IgG	10^{-11}	10^{9}
EIA (absorbance)	TSH	10^{-13}	10^{7}
EIA (fluorescence)	TSH	10^{-13}	10^{7}
EIA (chemiluminescence)	TSH	10^{-14}	10^{6}

IgG = immunoglobulin G
TSH = thyroid stimulating hormone

assay configurations come with limitations and no assay is suitable for all situations. While test strip assays are fast, easy and can be used outdoors, they are not very sensitive and not quantitative. The assays which are most sensitive and specific usually require well-controlled conditions, many washing and reagent solutions, and relatively sophisticated illumination and light detection optical components.

Both the RIA and EIA discussed previously are called heterogeneous assays or separation-required assays where the labeled and unbound components of the assay must be separated. However, a variant of the EIA was developed by Rubenstein et al. which required no separations. In this assay when the antigen binds to the antibody it inhibits the activity of the enzyme thereby reducing production of indicator molecules. This reaction is suitable for automation and uses standard enzymes to generate colored or fluorescent products. Chemiluminescent enzyme immunoassays assays have also been developed and these are quite sensitive. They require the same care as other EIA; however, there is no need for an external light source and, as a consequence, many of the problems associated with absorptive and fluorescent measurements are therefore precluded.

The objective of this project was to develop an exceptionally sensitive homogeneous immunoassay that would exhibit a very low false-positive rate and be robust enough that it could be rendered into a lateral-flow-like assay to enable automation. These are some of the criteria frequently mentioned as being necessary attributes of a field-deployable bioagent detector.

Technical Approach

Conceptual Application of Assay

The final rendering of the assay is envisioned to employ a chemiluminescent reaction that is initiated by the interaction of specific biological organisms with a special gelatin-coated material. Antibodies, specific for unique antigens found on the surfaces of biological structures (bacteria, spores, viruses, etc.), are contained in the coated material which then is treated with the sample medium. The biological organism of interest that comes in contact with the material will adhere to the coated surface where it will be interrogated by antibodies with binding sites specific for unique surface antigens on the organism. The antibodies are labeled with an enzyme that promotes the rapid oxidation of luminol to generate, in a completely light-tight environment, the bluish emission from the chemiluminescent reaction. The light is detected with a small photon counting device. Light detected beyond threshold limits activates a small alarm warning of the presence of the organism of interest.

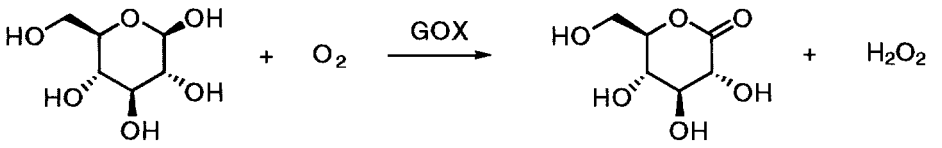
The detection of exceptionally small samples of biological material in an aerosol will be extremely difficult, especially in view of the time constraints related to field situations where many individuals risk exposure to highly active organisms. In such situations the concentration of biological material may be only tens of particles per liter of air so the analytical method must be capable of accurately analyzing a single particle in minutes to provide an adequate measure of protection. The

approach we follow addresses these issues of time, specificity, and accuracy by combining several well developed technologies. Firstly, we exploit the selective interactions between antibodies and their antigens to provide an exceptionally specific recognition component for detecting specific biological organisms. Secondly, we couple the antibody-antigen interaction to a chemiluminescence reaction that produces light only when a complimentary antibody attaches to the specific organism of interest. Thirdly, we make the chemiluminescent reaction occur catalytically so that the presence of a single biological cell or spore will produce hundreds of thousands of photons. And lastly, the light produced by the chemiluminescent reaction will be detected with a photon counting CCD detector. In the following sections major aspects of the sensing scheme are presented.

Proximal Pair Assay

The assay in this work is like many EIA systems in that it requires two antibodies, each of which must bind the antigen (or organism of interest). However, unlike standard heterogeneous EIAs where one antibody is used merely to capture the antigen and the other provides the indicator for signal transduction, the assay used in this work is homogeneous and employs two antibodies with each antibody carrying a necessary and complimentary part of the transduction mechanism. In this assay the two antibody populations are labeled with a different enzyme of a proximal-enzyme pair. These are pairs of enzymes that function together to produce a unique product that is not otherwise generated unless the two enzymes are located in very close proximity to one another. The limiting distance between the enzymes is a function of the reaction rates of the enzymes and the concentration of enzyme substrates in the medium.

There are a number of enzymes that constitute proximal pairs and a good example of one is glucose oxidase and horseradish peroxidase. Glucose oxidase (GOX) is a 160,000 Dalton flavin-containing enzyme that catalyzes the conversion of β-D-glucose into gluconolactone (Equation 4). In the process of oxidizing the sugar the GOX flavin is reduced, but in the presence of molecular oxygen the enzyme is converted back to its native state and oxygen is reduced to hydrogen peroxide (Equation 5). Under optimal conditions one GOX molecule can produce 20,200 molecules of gluconolactone per minute [Nakamura]. The GOX K_m, or the concentration of substrate required to produce half of the maximal reaction rate, is 33-35 mM for glucose and 0.95 mM for O_2. More importantly, the specific activity (30°C) for the consumption of O_2 is 172 μM/min/mg of GOX, meaning that 27520 moles of O_2 are consumed each minute by each mole of GOX. If the formation of hydrogen peroxide is efficient then a similar amount of H_2O_2 should be generated.



β-D-glucose gluconolactone (4)

$$GOX(FAD) \xrightarrow{glucose} GOX(FADH_2) \xrightarrow{O_2} GOX(FAD) + H_2O_2 \quad (5)$$

The second member of the pair is horseradish peroxidase (HRP), a 40,000 Dalton monomeric heme protein that converts H_2O_2 into water. However, the reaction requires a reducing agent to facilitate the reaction and in nature polyhydroxyphenols (AH_2) are the common electron donors. The K_m for H_2O_2 is on the order of 5 to 50 μM

[Kariya], and the specific activity of HRP is 295 μM/min/mg, equivalent to 11,800 moles of H_2O_2 being consumed each minute per mole of HRP [Shinmen].

The behavior of the indigenous enzyme is usually to react with hydrogen peroxide forming a energetic iron complex that subsequently reacts with aromatic compounds to form water (Equation 6) and radical aromatic intermediates ($AH\cdot$). The basic reaction involves the activation of the prosthetic heme (FeIII) followed by two sequential one electron oxidations of an aromatic substrate, AH_2 (Equations 7 and 8). In these two reactions after the first oxidation of AH_2, the oxidized product $AH\cdot$ diffuses out of the active site so that a second molecule of AH_2 can come to the active site and reduce heme back to its native state of Fe(III) [Dunford].

$$\text{HRP-Fe(III)} + H_2O_2 \rightarrow \text{HRP-Fe(IV)=O}\cdot + H_2O \quad (6)$$

$$\text{HRP-Fe(IV)=O} + AH_2 \rightarrow \text{HRP-Fe(IV)-OH} + AH\cdot \quad (7)$$

$$\text{HRP-Fe(IV)-OH} + AH_2 \rightarrow \text{HRP-Fe(III)} + H_2O + AH\cdot \quad (8)$$

By virtue of their mutual association with H_2O_2, these two enzymes, GOX and HRP, are a proximal pair. In the absence of adventitious peroxide, the two enzymes can induce the oxidation of an aromatic substrate with only glucose and molecular oxygen if the effective concentration of the respective substrates is sufficiently high. When the two enzymes are close to one another, and the first enzyme of the pair (GOX) is allowed to proceed at first order reaction conditions (i.e., 33-35 mM glucose and 0.95 mM O_2) then the effective concentration of H_2O_2 available to the second enzyme (HRP) will enable the second enzyme to carry out its reaction if the electron donor (AH_2) is also near its K_m value.

In an immunoassay, the conditions to promote this kind of proximal pair reaction should be easily achieved when the two enzymes are linked to antibodies that bind

the same antigen. In this way, a very dilute solution of the enzyme-labeled proximal pair enzymes can be concentrated at the surface of a small particle or antigen (a spore or vegetative cell) and provide the enzyme proximity necessary to ensure a concentration of substrate that allows the second enzyme of the pair to promote its own reaction series. However, the solution concentration of unbound antibody must be low to avoid generating a background concentration of second-enzyme substrate (H_2O_2). In the case of GOX and HRP, a third enzyme, unattached to any antibodies, can be included to the reaction volume to scavenge substrate (H_2O_2) generated in the bulk medium or that diffuses away from the antibody-bound antigen. Catalase (CAT), another heme enzyme, is a natural scavenger enzyme whose normal function in cells is to remove H_2O_2. It is a robust enzyme that converts two moles of H_2O_2 into two moles of water and a mole of O_2 [Equation 9]. The specific activity of CAT is one of the highest for any enzyme and in one minute a molecule of CAT oxidizes 980,000 molecules of H_2O_2 to water and O_2 [Claiborne].

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad (9)$$

Together with catalase and the proximal pair enzymes, a homogeneous assay can be developed that exploits the oxidizing ability of HRP and timely production of H_2O_2 by GOX. The two enzymes of the pair are separately attached through standard covalent linkages each to an antibody that recognizes a unique epitope on the surface of the antigen (see Figure 2). With judicious selection of the antibody populations so that they show no cross reactivity with each other and very little with epitopes on other antigens, the assay that results will demonstrate a very low rate of false-positive identifications because only when both enzyme-linked antibodies are attached to the specific organism of interest will a signal be generated. This two-antibody assay

facilitates highly specific antigen detection and reaps all the benefits from being homogeneous assay and free of troublesome separations during the assay. To achieve the greatest sensitivity for this type of assay and to simplify the detection methodology, the final catalytic step should result in luminescence. The substrate that provides the reducing equivalents to the activated HRP should produce light as a product of the reaction. The detection of photons in the absence of other light sources greatly simplifies the transduction process and minimizes many problems associated with low-level detection using other optical methods, such as absorption and fluorescence.

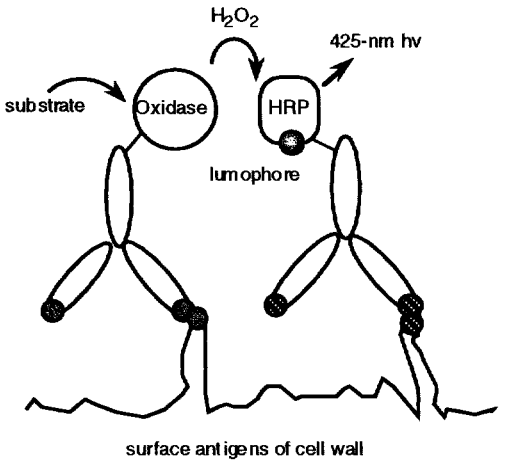


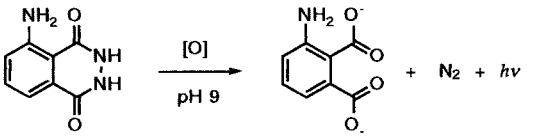
Figure 2. The antibody-tagged oxidase catalyzes the oxidation of substrate to form hydrogen peroxide. This compound is a substrate for the antibody-tagged HRP and reduction of the peroxide results in very rapid oxidation of the lumophore to produce light. Because the only source of peroxide is the oxidation of substrate, the concentration of the HRP substrate (peroxide) will only be significant when the two enzymes are juxtaposed.

Chemiluminescent Transduction Mechanism

There are several different chemical systems commonly used in assays based on chemiluminescence, but a very simple reaction between comparatively robust and inexpensive chemicals is the oxidation of 3-aminophthalylhydrazide (**1**, luminol). The reaction produces a bluish-white light that can be seen with the human eye.

The reaction of luminol with oxidizers has been studied for many years, but only recently have details of its specific mechanism been reported. The current interest is undoubtedly due to the potential use of the reaction as an indicator in sensitive assays for a variety of biological, organic and inorganic analytes. The overall conversion of luminol into photons and chemical products is shown in Chart 1, with luminol undergoing oxidation in basic media to form 3-aminophthalic acid (**2**), molecular nitrogen, and blue light. The reaction progresses with the most intense luminescence occurring in basic solutions with the pH ranging between 11 and 12. Solutions too basic inhibit the reaction and pH's between 8 and 11 merely cause the reaction to proceed at a slower rate.

Chart 1

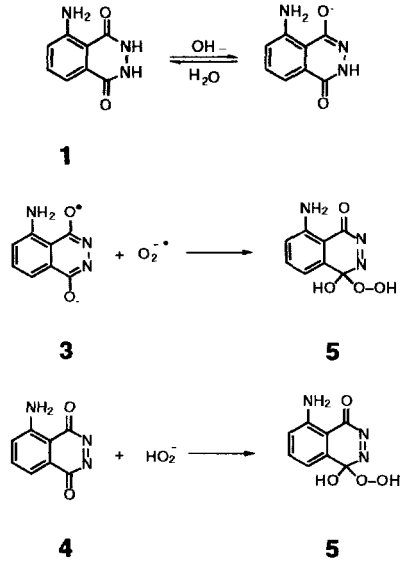


1 **2**

Careful study shows that decomposition of the hydroperoxide (**5**, Chart 2) produces the electronically excited phthalic acid anion which is the actual luminescent species. In aqueous media **1** is in equilibrium with its tautomer, but in basic solution the iminol is ionized as the anion. Pulse radiolysis and kinetic studies show in basic media that the ionized luminol undergoes a series of one electron oxidations to give intermediates **3** or **4**, depending on the specific conditions of the media. Each intermediate reacts with an active form of oxygen (hydroperoxide, HO_2^-; or superoxide, $O_2^{-\cdot}$) giving the hydroperoxide, **5**. pH dependent deprotonation of **5** causes release of diatomic nitrogen and formation of the diacid in an electronically excited state.

Relaxation of **2*** to the ground state through fluorescence provides visible light with a λ_{max} at 430 nm. The bandwidth of the emission peak at half height is about 65 nm. The luminescence efficiency of the reaction is described by Equation 10, where Φ_f is the fluorescence quantum efficiency of the emitter **2**, Φ_r is the reaction product yield of **2**, Φ_{ce} is the chemical excitation efficiency, and Φ_{ch} is the chemical quantum yield, or the overall efficiency for the production of light from hydrazide **1**. In aqueous media representative values of these terms are shown in Table 2.

Chart 2



$$\Phi_{ch} = \Phi_f \Phi_r \Phi_{ce} \quad (10)$$

Table 2. Quantum and Chemical Efficiencies of Luminol Chemiluminescence Reaction*

Φ_{ch}	chemical quantum yield	0.004
Φ_f	fluorescence quantum efficiency of the emitter	0.4
Φ_r	product yield	0.5
Φ_{ce}	chemical excitation efficiency	0.02

*Oxidation of luminol in aqueous media with NaOCl as the non-catalytic oxidant.

The chemiluminescence reaction cascade can be initiated with strong oxidizing reagents (bleach, i.e., NaOCl) that directly interact with **1**, or milder oxidants that do not themselves promote the reaction but, when used in the presence of a catalyst, the oxidation of **1** progresses rapidly with the production of light. For the reactions that occur only in the presence of a catalyst, oxidants such as hydrogen peroxide or other hydroperoxides are commonly employed. The catalytic agent is often a transition metal ion, but for controlled sensitive measurements, iron porphyrins (deuterohemin) or heme-based enzymes (horseradish peroxidase or microperoxidase) are the ideal catalysts. The enzyme-based reactions are normally conducted under minimally basic conditions (pH 9) to avoid protein denaturation; however, these bio-catalysts provide the additional benefit that enhancers can be used to increase the luminosity of the reaction by as many as two orders of magnitude. The cause of the enhancement is known [Diaz], and phenolic compounds [Sanchez], such as *p*-iodophenol, are typical examples of luminescence enhancers [Goodwin]. The relatively stable radicals derived from these halophenols by the enzymatic (HRP) one electron oxidations shown in Equations 7 and 8 are the actual oxidizing agents of luminol. These radicals diffuse out of the enzyme active site and into the bulk medium to subsequently oxidize luminol. The enzyme reaction rate for the oxidation *p*-iodophenol is 10^7 mol^{-1} s^{-1}, nearly three orders of magnitude faster than chemical oxidation of **1**. When enhancers are used, the rate of light emission is much greater, increasing by 10^2 to 10^4 times the rate when just luminol is used. Without the addition of enhancers the reaction progresses more slowly over tens of minutes.

The Biological Organism and Its Antigens

The surfaces of bacteria, germinate and sporulate forms, fungi, and viruses are coated with proteins and sugars that possess antigenic activity. Even though the

surface area of these organisms is obviously very small (3-10 μ^2), they still contain unique proteins that can be used as immunochemical indicators for a particular species of organism.

The dimensions of the Fab fragment can be used to estimate the number of antibodies that might possibly bind to the surface of a bacterium. If we use only one Fab region to bind one antigenic site on the cell wall, and we assume that the antibody fragment will situate itself orthogonal to the cell surface to maximize packing density, then the area required by the fragment at the surface of the cell is roughly 12 nm^2. Bacteria come in a variety of shapes and sizes, but their surface area spans a range between 3-10 x 10^6 nm^2. This area provides the space for an estimated 10^5 Fab fragments to bond epitopes at the surface of a cell.

The peroxidase enzymes are exceptionally active; one molecule of enzyme can reduce 12000 molecules of peroxide per minute. The glucose oxidase is also an active enzyme as one molecule will, each minute, oxidize about 25000 molecules of glucose and produce 25000 molecules of hydrogen peroxide. Given that there are potentially thousands of sites to which antibodies can bind on a single organism (using the *E. coli* example of 10^5 OmpA proteins) it is conceivable that 10^7 catalytic cycles could occur in one minute. This enzymatic activity would amount to generating perhaps as many as 10^7 photons per minute with the luminescence lasting for more than a minute as the substrates are consumed. The signal could be integrated over time to produce a response necessary to exceed a preset threshold need to initiate an alarm.

Results

The initial effort focused on selecting the best candidate enzymes for the proximal-pair system. The results of this evaluation are listed in Table 3 and represent

important factors considered in selecting enzymes for the assay. Although no one factor itself determined the selection of enzymes, clearly the stability of the enzyme, its robustness, and a relatively high turnover number were of preeminent concern. Consequently, despite its high mass (160 kD), glucose oxidase (GOX) was selected as the enzyme of the proximal pair to produce the hydrogen peroxide for the second enzyme of the pair, horseradish peroxidase (HRP).

Table 3. Common peroxide producing enzymes and some of their characteristics.

EC No.	enzyme	mol mass[1]	cofactor[2]	multiple[3]	MA[4]	source
1.1.3.4	Glucose Oxidase	150-180(gly)	FAD(t)	2H	27520	*Aspergillus niger*
1.1.3.6	Cholesterol Oxidase	65.1	FAD(n)	1	388	
1.1.3.7	Aryl Alcohol Oxidase	71(gly)	FAD	1	2772	*Pleurotus sajor-caju*
1.1.3.9	Galactose Oxidase	72 (gly)	Cu	1	8352	*Dactylium dendroides*
1.1.3.13	Alcohol Oxidase	300(gly)	FAD(c)	4H	1980	*Pichia sp*
1.1.3.15	Glycolate Oxidase	172	FMN	4H	619	Spinach
1.1.3.17	Choline Oxidase	66	FAD(c)	1	891	*Alcaligenes* s
1.2.3.4	Oxalate Oxidase	120	Cu	2H	46	sorghum
1.3.3.6	Acyl-CoA Oxidase	415	FAD	6H	1702	Rat liver
1.4.3.1	D-Aspartate Oxidase	37	FAD(n)	1	296	*Octopus vulgaris*
1.4.3.2	L-Amino Acid Oxidase	132	FMN	2	71.3	Pit Viper
1.4.3.3	D-Amino Acid Oxidase	76.4	FAD(t)	2H	13373	*Rhodotorula gracilis*
1.4.3.6	Copper Amine Oxidase	114	Cu	2H	4.4	*Saccharomycescerevisiae*
1.4.3.11	L-Glutamate Oxidase	28.2				*Streptomyces* s
1.7.3.3	Urate Oxidase	73			891	*Candida* s

1. molecular mass in kilodaltons, gly = glycopeptide; 2. enzyme prosthetic group; 3. multiplicity of enzyme: 2H = homodimer, 4H = homotetramer; 4. molar activity = moles product/mole enzyme/min.

The HRP catalyzed oxidation of luminol with H_2O_2 was studied in solution with several potential enhancer molecules to boost the overall chemical quantum efficiency. Several *p*-substituted phenols were tested (I, Br, Cl, H_2N, CH_3, and CO_2H) with the halogenated compounds showing the largest increase in luminescence. The luminescence spectrum is shown in Figure 3a along with a plot of the light intensity

produced from the decomposition of the oxidized luminol as a function of the reaction's progress. In the latter graph (3b), the effect of the enhancers is shown by the increase in the luminescence during the first several minutes of the reaction in the order of I- > Br- > Cl- *p*-substituted phenols. Similarly, the quantity of light produced over the course of the reaction follows the order: I > Br > Cl. Regardless of the site of oxidation, the oxidation of luminol in the presence of the enhancers is significantly more efficient. The rate of light production is a function of the enhancer concentration, although the kinetics of the reaction have not yet been studied.

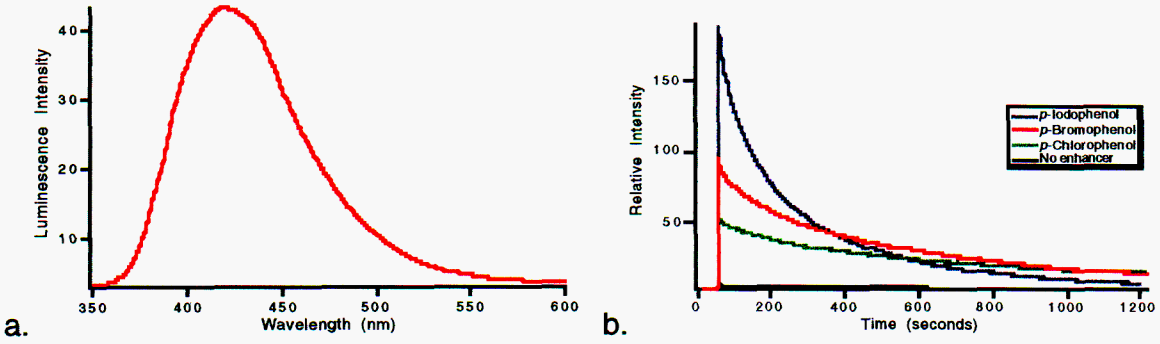


Figure 3. (a) The luminescence spectrum luminol oxidation with HRP and H_2O_2 in pH 8.5 buffer at room temperature. (b) The luminescence intensity plotted against the reaction time for equimolar solutions of three *p*-substituted halophenol (I, Br, Cl,) enhancers. A fourth curve (nearly at the baseline) is that of a control reaction performed in the absence of any enhancer. Integration of the curves shows that iodophenol produces the most light over the period of the reaction.

In order to evaluate the reaction conditions necessary for a specific bioorganism-promoted proximal pair reaction, the antibody reaction with antigen was first modeled using micron-size affinity chromatography beads coated with avidin. Avidin is a protein that has four binding sites and binds the relatively small molecule biotin (MW 244) with an association constant, K_a, of 10^{15} M^{-1}. The strength of this association is virtually as strong as a covalent linkage and so the biotin-avidin linkage is often used to very mildly couple proteins with other molecules. Using biotinylated HRP and GOX the antibody-antigen reaction could be mimicked and the reaction

conditions for developing the chemiluminescence could be determined. These surrogate reactions would also save valuable bacterial antibody stocks for assay development with antigen. The general reaction is shown schematically in Figure 4. The appropriate substrates were provided for each individual enzyme system (4a and 4b) so that activity would be evidenced by oxidation of luminol with production of light. As both sets of enzyme-complexed beads were active, a third series was produced by treating the avidin-coated beads with mixtures of the biotinylated HRP and GOD in varying ratios (4c). In this last series, addition of a solution containing glucose, iodophenol, and luminol to the GOD/HRP-complexed beads resulted in the production of light, as would be expected from the proximal pair assay. In Figure 5 is a CCD sensor image obtained with a 5-second integrated exposure of the beads viewed through a microscope as they luminesced.

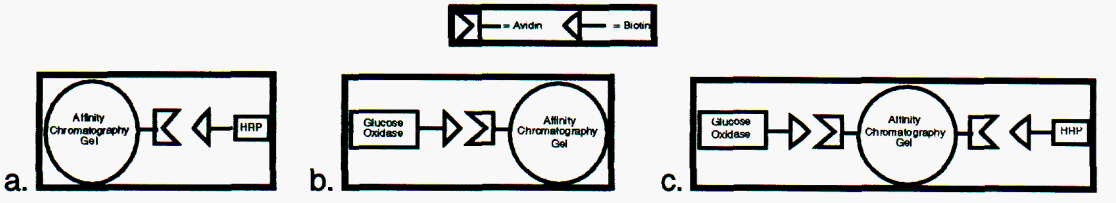


Figure 4. A schematic depicting the series of reactions with avidin coated beads and biotinylated enzymes. Avidin binds biotin non-covalently but with an association constant of 10^{15}. (a) The reaction of biotinylated HRP with avidin coated beads, (b) the reaction of biotinylated GOD with avidin coated beads, (c) the reaction of mixtures containing varying molar ratios of biotinylated HRP and biotinylated GOD with avidin coated beads.

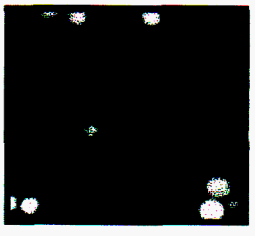


Figure 5. An image of luminescing beads produced by a Pixel-211 CCD camera with a 192 x 164 pixel array. The image was collected during the oxidation of luminol with avidin-coated beads treated with biotinylated HRP. The beads were viewed through a microscope with a 40X objective. The sensor was exposed for 5 seconds.

With the biotin-avidin reaction of the two proximal pair enzymes successful, the assay development progressed to bacteria-size antigens and enzyme-linked antibodies. Polystyrene beads (between 0.8μ and 3.5μ diameter) coated with goat-antirabbit antibody were used as a bacterial surrogate. The diameter of the beads is within the size range of most bacteria. A solution of the antigen-linked beads in pH 8 buffered glucose (1M) containing iodophenol and luminol (100 μM each) was prepared and a 50 μL aliquot was placed on a square (4 cm^2) of Kodak lens paper fixed to a microscope slide. To the wet paper was then added 10 μL of HRP- and GOX-linked rabbit-antigoat antibodies (10 μg/mL in pH 7 PBS). Within a minute, the beads could be seen with the naked eye luminescing in the dark through a microscope. In this experiment no catalase was used to scavenge excess peroxide.

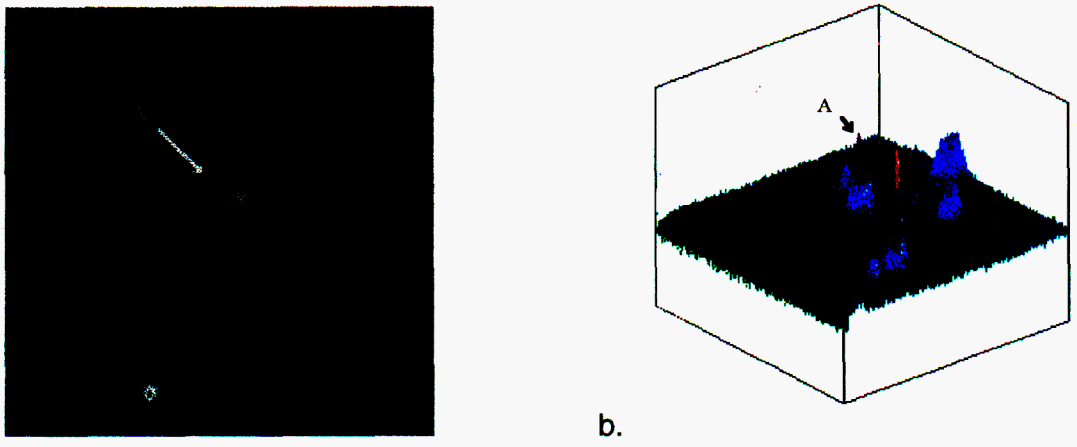


Figure 6. (a) A 16 bit digitized CCD image of luminescing beads produced by a Apogee CCD camera with a 512 x 512 pixel array. The image was generated by a 2 second shuttered exposure of antigen-labeled beads treated with GOD- and HRP-linked antibodies in a solution containing glucose and luminol. (b) 3D rendering of data from the 2D black-and-white image at left. The green plane represents the 512 x 512 pixel array and the vertical element corresponds to the 16-bit pixel value.

Pixel Size: 24 x 24 μ
Array Size: 12.3 x 12.3 mm
Pixel Capacity: 350,000 e^-
Quantum Efficiency (410 nm): 0.80

Number of Pixels in A: 1124
Integrated Pixel Value (bits): 1.63×10^7
Electrons per Bit (16-bit pixel value): 5.34
Integrated Photon Count: 8.77×10^7 s^{-1}

Figure 6a shows the digitized CCD image of the luminescing beads collected with an Apogee CCD camera cooled to minus 10°C with built-in thermoelectric coolers. Figure 6b is a 3D rendering of the data. Analysis of the detector response indicates that a single randomly chosen bead (labeled A in Figure 6) produced a signal from which 8.77 x 10^7 photons per second were detected during the initial five minutes of the antibody-antigen reaction. The array in this detector is back-thinned and has an effective quantum efficiency of 0.8 at 410 nm. The responsiveness of the back-thinned arrays is much better in the blue region than are standard CCD detectors and this is shown in Figure 7. The luminescence spectrum of luminol is shown in relation to the spectral dependence of the detector sensitivity for back-thinned (UV-sensitive) and standard CCD arrays.

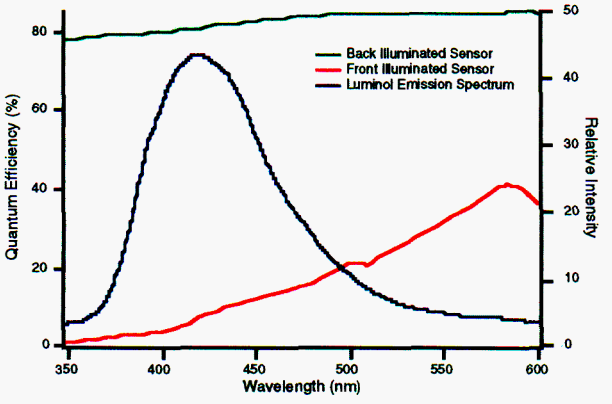


Figure 7. The spectral responsivity of back-thinned and standard CCD detectors is shown in relation to the chemiluminescence generated by the HRP-GOX-luminol reaction.

Conclusions

Clearly, the experimental results described in this work demonstrate that the chemiluminescent proximal-pair assay is ideally suited for antigen detection using a homogeneous assay. Unfortunately, the assay was not performed with actual bacterial spores because only one enzyme had been linked to antibodies by the close

of the project. Nevertheless, a conceptual model of an assay adapted for use in a multi-assay hand-size detector is shown in Figure 8.

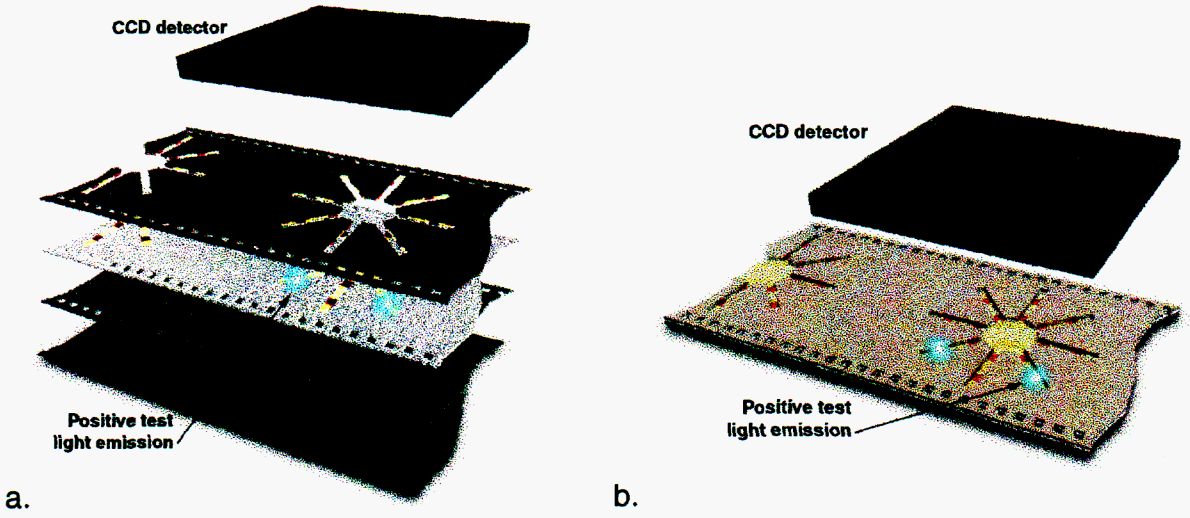


Figure 8. (a) A lateral flow assay is shown built into several layers of assay material organized in a film-like arrangement. The central circle represents a well where antigen-containing fluid is deposited . Liquid migrates radially down the channels containing the proximal-pair antibodies (red dots). If the antigen traveling down the channel is not complimentary to the antibody, it continues past the second antibody to a sink (not shown). (b) Shows the chemiluminescence developing from an antigen-antibody interaction.

A lateral flow assay is shown built into several layers of assay material organized in a film-like arrangement. The central circle represents a well where antigen-containing fluid is deposited. As in standard lateral flow assays, the liquid migrates radially by capillary action down the channels containing the lyophilized/stabilized proximal-pair antibodies (red dots). At the entrance to each channel is located the HRP-linked antibody for a particular antigen. A slow release dam on the proximal side of this antibody allows the pool of antigens in the central well time to bind to their respective antibodies. When the dam has dissolved, the concentrated antibody-linked antigen migrates down the channel to the second antibody of the proximal pair. If the antigen is not complimentary to the antibody, it

continues down the channel to a sink (not shown). However, if it is the correct antigen, it binds to the GOX-linked antibody attached to the channel matrix. The two enzymes attached to their immobilized antibodies then initiate the chemiluminescent reaction (blue spot in Figure 8).

The assay could be built into a film-like strip such that each strip could contain tens of assay. The strips could then be wound, much like photographic film, and placed into containers that, like film, would be exposed to fluids or atmosphere to collect samples for analysis. Figure 9 shows a conceptual model of a hand-size detector which would hold the film, like a camera, and perform the assay following exposure of the film. The CCD in the detector would read the assay and display the results on the camera's LCD screen. In addition, the data could be transferred to memory card in the camera and to a magnetic band built into the assay film strip. This configuration of the assay would allow any material collected to remain on the film to be retrieved and interrogated by other analytical methods.

Figure 9. The Personal Detection System for BW agents. The hand-size camera contains assay film complete with all the elements necessary to test for many agents. The CCD operates on-demand to minimize power requirements. The unit could be developed to accept aqueous solutions or collect aerosol samples. Each film cassette would contain all the reagents required for the assay, even water for dry or aerosol samples.

Future Funding

The concern that a terrorist attack may occur in the U.S. or against U.S. citizens and interests overseas has many agencies within the federal government pursuing various programs to mitigate the potential of such an event. Many agencies are also developing new technology to rapidly detect and identify biological warfare agents in the case of an actual event. We are pursuing the possibility of future funding with several of these government agencies.

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